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Supplemental material

Iron starvation triggers the stringent response and induces amino acid biosynthesis for bacillibactin production in *Bacillus subtilis*

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Description of microarray analysis:

I) Microarray preparation. Microarrays containing 70-72 bps oligonucleotides specific for 4150 *B. subtilis* genes were prepared as described by Lulko *et al.* (10).

II) Cell growth, RNA isolation, cDNA synthesis, cDNA labeling and slide hybridization. The *B. subtilis* strain ATCC 21332 (5) was cultivated in minimal medium (14) (supplemented with 0.45 % [w/v] glucose and 3.5 mM sodium glutamate) without citrate and iron as described previously (11). A preculture grown in iron-depleted medium was inoculated into fresh medium either without addition of iron (for continued iron starvation) or with 10 µM $FeSO_4$ (for iron repletion) to an initial OD_{600} of 0.04. At an OD_{600} of 0.35, the cells were harvested and the total RNA was prepared as described previously (6).

The iron limitation in the iron-depleted cultures was confirmed by an additional growth experiment (Fig. S1): the iron-depleted preculture was inoculated into the main cultures (either iron-depleted or iron-repleted) to an initial OD_{600} of 0.02, and samples were taken at various time points during growth to determine the bacillibactin content in the culture supernatants by using the Arnow test (1) for specific catechol detection and a standard

calibration curve based on the absorption of Arnow-treated 2,3-dihydroxybenzoic acid at 510 nm.

The RNA samples were further purified by using the High Pure RNA isolation kit (Roche Molecular Biochemicals) including DNaseI treatment. The RNA quality was checked with an Agilent 2001 bioanalyzer (Agilent Technologies). For cDNA synthesis, 10 to 20 µg of total RNA was incubated with 2 µl of random nonamers (1.6 µg/µl) in a total volume of 18 µl for 5 min at 70 °C before annealing was allowed by cooling down to room temperature for 10 min. After addition of 6 µl 5 × Superscript III buffer, 3 µl 0.1 M DTT, 1.2 µl 25× amino-allyl-modified dUTP/ nucleotide mix and 1.8 µl reverse transcriptase (Superscript III), reverse transcription was performed in a total volume of 30 µl for 16 h at 42 °C. Next, the RNA was degraded by adding 3 µl of 2.5 M NaOH followed by incubation for 15 min at 37 °C. The solution was neutralized with 15 µl of 2 M HEPES (free acid). The cDNA was purified using GFX columns (Amersham Biosciences) according to the instructions of the manufacturer (with the exception that 80% ethanol was used as wash buffer) yielding concentrations between 30 to 120 ng/µl. Labeling of the amino-allyl-modified cDNA was performed with Cy3 and Cy5 fluorescence dyes and subsequent purification was carried out according to the manufacturers protocol yielding concentrations of 0.5 - 2.0 pg/µl of labeled cDNA. The labeling was carried out with cDNA obtained from three independent RNA preparations of both iron depleted and repleted cell cultures and was always performed twice (with Cy3 and Cy5, respectively → "DYE SWAP") which allowed the performance of six competitive hybridizations using Cy3- and Cy5-labeled cDNAs in both combinations to avoid labeling-specific effects. The amino-allyl-modified cDNA was purified with the Cyscribe GFX purification kit (Amersham Biosciences) using 80% ethanol as wash buffer and 0.1 M sodium carbonate (Sigma-Aldrich) pH 9.0 as elution buffer. For competitive hybridization, the labeled cDNA samples were mixed, dried by vacuum rotation and boiled in 5 µl water for 2 min. After incubation on ice for 2 min, the samples were resolved in 30 µl preheated Ambion SlideHyb #1 hybridization buffer (Ambion Europe Ltd., Huntingdon, UK) at 68 °C for 5 min. After spinning at maximum speed, the samples were applied to the preheated microarray slides and hybridization took place in the dark at 48 °C for at least 16 h. The slides were washed with 40 ml 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate; pH 7.0) and 0.5 % SDS for 5 min, then twice with 40 ml 1 × SSC and 0.25 % SDS for 5 min, then with 40 ml 1 × SSC and 0.1 % SDS for 5 min. The slides were dried by centrifugation (at 805 × g for 2 min at room temperature) and were subsequently applied to a convocal laser scanner for signal detection.

III) Data analysis. Six microarrays in total were used for the data analysis. The arrays were scanned in two channels specific for either Cy3 or Cy5 fluorescence detection. The spot signals of the images were quantified by using the Array-Pro® Software (Version 4.5.1.48; Media Cybernetics, Inc.). Normalization of the spot intensity values was performed with both the PreP and MicroPreP Software (7, 15). Twelve normalized signal values were obtained for each gene, as all genomic fragments were spotted in duplicate on the arrays. For a statistical evaluation of the data, a CyberT analysis was performed to calculate the mean induction ratios and the Bayesian standard deviations (Bayes.p values) of all normalized signal intensities (3) which is summarized in Table S1. Only genes with a highly homogeneous expression pattern (Bayes.p < 10^{-3}) were selected to identify significantly overrepresented functional categories using the FIVA software (4). Due to the stringent significance parameters that were chosen to select these data, it was possible to include genes showing moderate changes in expression. Therefore, the upper and lower fold-cutoff for gene induction and repression was set at 1.4 and 0.7, respectively. Affected genes falling in significantly enriched functional or regulatory groups identified by the FIVA program are listed in Table S2.

Description of dot blot analysis:

An additional independent growth experiment with iron-depleted and iron-repleted cell cultures was carried out as described for the microarray analysis (see above). Total RNA was isolated from these cell cultures according to the acidic phenol method (8). Two micrograms of total RNA were dotted onto a nylon membrane (Amersham Biosciences) using a dot blot apparatus (Bio-Rad Laboratories GmbH) and hybridized after UV-crosslinking with digoxigenin-labeled antisense RNA probes specific for *citZ*, *citB*, *yclM*, *hom*, *yoaD*, *gltA*, *rpsP*, *lpdV*, *yurO*, *ald*, *dhbF* and *feuA* mRNAs. The riboprobes were synthesized by *in vitro* transcription using T7 RNA polymerase (Ambion Inc.) and PCR products of the corresponding genes containing a T7 promoter extension that was introduced by the primers listed in Table S3. After hybridization, the filters were treated with a digoxigenin-specific antibody fragment conjugated with an alkaline phosphatase (Roche) and ECF as a chemiluminescence substrate (Amersham Biosciences). The hybridization signals were detected using the blue-chemiluminescence channel of a Storm860 fluorescence imager.

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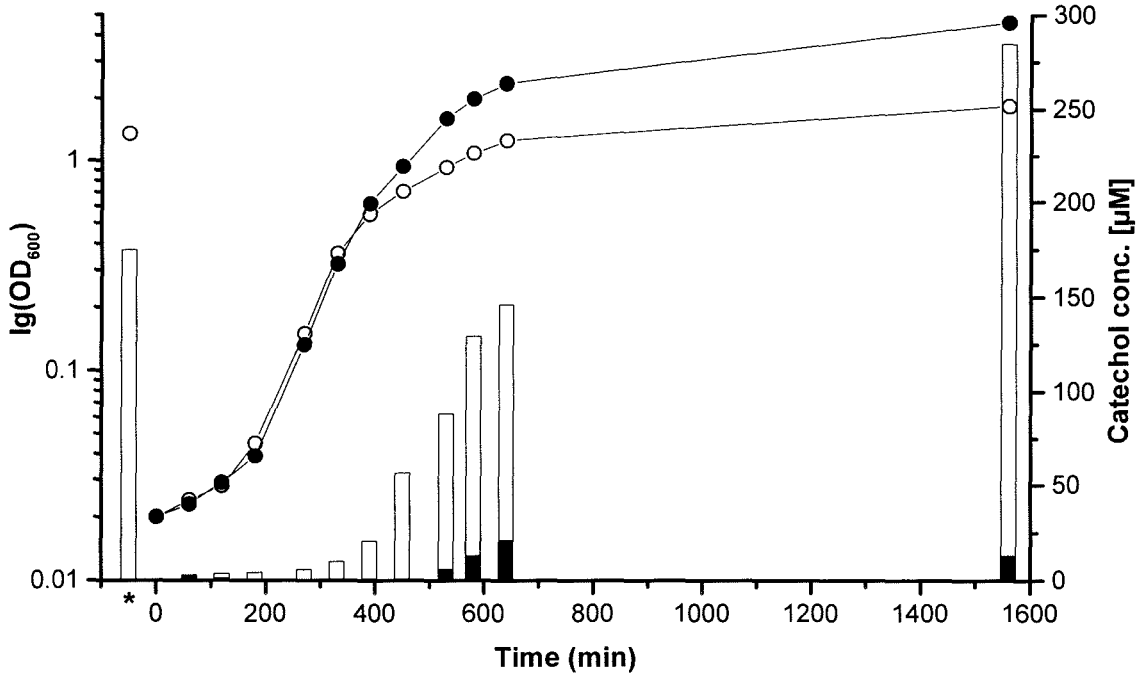

FIG. S1. Growth curves and catechol siderophore secretion of *B. subtilis* ATCC 21332 cultured in iron-depleted and iron-repleted minimal medium under agitation (220 rpm) at 37 °C. A preculture was grown in iron-depleted medium and the OD_{600} (open circle) and the catechol siderophore content (open bar) were determined after 16 h (indicated by a star on the x-axis). Cells of the preculture were inoculated into fresh medium that was either iron-depleted or contained 10 µM $FeSO_4$ for iron repletion. The growth (OD_{600}) of the iron-depleted (open circles) and iron-repleted cultures (filled circles) was monitored (starting at 0 min on the x-axis). After 60 min of growth, samples were taken at several time points and the catechol siderophore content was determined in the supernatants of the iron-depleted (open bars) and iron-repleted (filled bars) culture by the Arnow test (1) and by using a standard calibration curve for 2,3-dihydroxybenzoic acid.

TABLE S2. Cluster analysis of *B. subtilis* ATCC 21332 genes affected in their expression during iron starvation in minimal medium

Category	Up-Regulation (> 1.4)		Down-Regulation (< 0.7)	
	gene	function	gene	function
Functional				
Siderophore biosynthesis, iron transport and release	*dhbA*[1]	2,3-dihydro-2,3-DHB DHG		
	dhbC[1]	isochorismate synthase		
	dhbE[1]	2,3-DHB-AMP ligase		
	dhbB[1]	isochorismatase/ ArCP		
	dhbF[1]	BB NRPS		
	feuA[1]	ferri-BB binding protein[f,g]		
	feuB[1]	ferri-BB permease[f,g]		
	feuC[1]	ferri-BB permease[f,g]		
	ybbA[1]	put. esterase		
	yuiI[1]	ferri-BB trilactone hydrolase; *designated besA*[f]		
	fhuB[1]	ferri-hydroxamate permease		
	fhuG[1]	ferri-hydroxamate permease		
	fhuD[1]	ferri-hydroxamate binding protein		
	yhfQ[1]	put. iron(III) dicitrate-binding protein		
	yxeB[1]	put. ABC transporter (binding protein)		
	ywbL[1]	put. iron permease (FTR1 family)[c,g]		
	ywbM[1]	unknown, suggested role in iron transport[g]		
	ywbN[1]	put. peroxidase, suggested role in iron transport[g]		
Amino acid biosynthesis	*lysC*	aspartokinase II	*hisC*	histidinol-phosphate AT
	yclM	aspartokinase III	*hisZ*[5]	ATP phosphoribosyltransferase regulatory subunit
	hom	homoserine dehydrogenase	*hisA*[5]	1-(5-Phosphoribosyl)-5-[(5-phosphoribosylamino) methylideneamino]-imidazole-4-carboxamideisomerase
	thrB	homoserine kinase	*hisI*[5]	histidine biosynthesis bifunctional protein HisIE
	thrC	threonine synthase	*hisB*[5]	imidazoleglycerol-phosphate dehydratase
	cysE	serine acetyltransferase	*hisF*[5]	imidazole glycerol phosphate synthase subunit
	yjcI[6]	put. cystathionine γ-synthase	*hisH*[5]	imidazole glycerol phosphate synthase subunit
	yjcJ[6]	put. cystathionine β-lyase	*hisD*[5]	histidinol dehydrogenase
	yxjG	similar to methionine synthase MetE	*hisG*[5]	ATP phosphoribosyltransferase
	yitJ[6]	put. methylene-THF reductase	*argC*	N-acetyl-gamma-glutamyl-phosphate reductase
	yoaD[2),6)]	put. 3-Phosphoglycerate DHG	*argJ*	ornithine and amino-acid acetyltransferase
			argB	acetylglutamate kinase
			argH	argininosuccinate lyase
			gltA	glutamate synthase [NADPH] large chain
			gltB	glutamate synthase [NADPH] small chain
			trpA	tryptophan synthase alpha chain
			trpB	tryptophan synthase beta chain
			trpC	indole-3-glycerol phosphate synthase
			trpD	anthranilate phosphoribosyltransferase
			trpE	anthranilate synthase component I
			trpF	N-(5'-phosphoribosyl)anthranilate isomerase
Nucleotide biosynthesis	*yerA*	put. adenine deaminase	*guaA*	GMP synthetase
			nrdF	ribonucleoside-diphosphate reductase (minor subunit)
			prs[3b)]	phosphoribosylpyrophosphate synthetase
			purD[5]	phosphoribosylglycinamide synthetase
			purF[5]	glutamine phosphoribosylpyrophosphate amidotransferase
			purH[5]	phosphoribosylaminoimidazole carboxy formyl formyltransferase / inosine-monophosphate cyclohydrolase

			purM[5]	phosphoribosylaminoimidazole synthetase
			purN[5]	phosphoribosylglycinamide formyltransferase
			purS[5]	required for phosphoribosylformylglycinamidine synthetase activity
			pyrAA[5]	carbamoyl-phosphate synthetase (glutaminase subunit)
			pyrAB[5]	carbamoyl-phosphate synthetase (catalytic subunit)
			pyrC[5]	dihydroorotase
			pyrD[5]	dihydroorotate dehydrogenase (catalytic subunit)
			pyrF[5]	orotidine 5'-phosphate decarboxylase
			pyrH[3b]	uridylate kinase
			pyrK[5]	dihydroorotate dehydrogenase (electron transfer subunit)
			udk	uridine kinase
Translation and ribosomal structure organization	*cysS*	cysteinyl-tRNA synthetase	*frr*[3b]	ribosome recycling factor
			infB[3b]	translation initiation factor IF-2
			infC[3b]	translation initiation factor IF-3
			rbfA	ribosome-binding factor A
			rplL[3b]	50S ribosomal protein L7/L12
			rplT[3b]	50S ribosomal protein L20
			rpmA[3b]	50S ribosomal protein L27
			rpmB[3b]	50S ribosomal protein L28
			rpmE[3b]	50S ribosomal protein L31
			rpmF[3b]	50S ribosomal protein L32
			rpmH	50S ribosomal protein L34
			rpsD[3b]	30S ribosomal protein S4
			rpsI[3b]	30S ribosomal protein S9
			rpsO[3b]	30S ribosomal protein S15
			rpsP[3b]	30S ribosomal protein S16
			rpsR[3b]	30S ribosomal protein S18
			truA[3b]	tRNA pseudouridine synthase A
			ybxB	put. 16S rRNA G1207 methylase
			ylxQ[3b]	put. ribosomal protein
			ymcB	put. tRNA modification enzyme
			yugI[3b]	put. polyribonucleotide nucleotidyltransferase
Regulatory				
Fur-dependent genes[c]		[1] all indicated above		
	ydbN	unknown		
	ybbB	put. transcriptional regulator		
	yoaJ	put. endoglucanase		
	yfhC	put. nitroreductase		
	ykuN	put. flavodoxin		
	ykuO	put. glycoside hydrolase		
	ykuP	put. flavodoxin		
CodY-dependent genes[d]		[2] all indicated above		
	dppB	dipeptide ABC transporter	*citB*[h]	aconitase
	ureA[4]	urease (γ subunit)	*gltT*	H+/Na+-glutamate symport protein
	bkdR	transcriptional activator	*ydcL*	put. integrase
	lpdV	dihydrolipoamide dehydrogenase	*ykaA*	unknown
	bkdAA[3a]	2-oxoisovalerate dehydrogenase (α subunit)		
	bkdAB	2-oxoisovalerate dehydrogenase (β subunit)		
	bkdB[3a]	lipoamide acyltransferase		
	rapA[4]	response regulator aspartate phosphatase		
	gabP[4]	gamma-aminobutyrate permease		

	ycgA	unknown		
	amhX	amidohydrolase		
	yoaC[6]	put. xylulokinase		
	yfmI	put. macrolide-efflux transporter		
	yfmJ	put. quinone oxidoreductase		
	ykwB	unknown		
	yuiA	unknown		
	yvdA	put. carbonic anhydrase		
	yurJ[4]	put. ABC-transporter (ATP- binding protein)		
	yurL[4]	put. ribokinase		
	yurM[4]	put. ABC-transporter (permease)		
	yurN[4]	put. ABC-transporter (permease)		
	yurO[4]	put. ABC-transporter (substrate binding)		
	yurP[4]	put. glutamine-fructose-6-phosphate AT		
RelA-dependent stringent response genes of described negative regulation[e]		[3a] all indicated above		[3b] all indicated above
			ssb	single-strand DNA-binding protein
			tig	trigger factor (prolyl isomerase)
			ylaG	put. GTP-binding elongation factor
			trmU	probable tRNA methyltransferase
			murD	UDP-N-acetylmuramoylalanyl-D-glutamate ligase
			qoxD	cytochrome aa3 quinol oxidase (subunit IV)
			ylbN	unknown
			ylxP	unknown
			yrvE	put. ssDNA-specific exonuclease
RelA-dependent stringent response genes of described positive regulation[c]		[4] all indicated above		
	vpr	minor extracellular serine protease		
	ald	L-alanine dehydrogenase		
	phrA	phosphatase (RapA) inhibitor		
	spoVG	required for spore cortex synthesis		
	spo0A	sporulation two-component response regulator		
	gspA	general stress protein		
	ytzE	put. transcriptional regulator (DeoR family)		
	ypiB	unknown		
	yetH	put. glyoxalase family protein		
	ykzF	unknown		
RelA-independent genes affected negatively during stringent response[e]				[5] all indicated above
RelA-independent genes affected positively during stringent response[e]		[6] all indicated above		
	yoaB	put. α-ketoglutarate permease		

Abbreviations: ArCP, Aryl carrier protein; AT, aminotransferase; BB, bacillibactin; DHB, 2,3-dihydroxybenzoate; DHG, dehydrogenase; NRPS, nonribosomal peptide synthetase; put., putative; THF, tetrahydrofolate.

[c] according to supplemental reference (2).

[d] according to supplemental reference (12).

[e] according to supplemental reference (6).

[f] according to supplemental reference (11).

[g] according to supplemental reference (13).

[h] CodY has no effect on *citB* expression in minimal medium (see supplemental reference [9]).

TABLE S3. Primers used for riboprobe synthesis (the T7 promoter extension is underlined)

Gene	Primer	Sequence (5' → 3')
citZ	citZ_for_T7	GATATCGATGATTTGACAGAGAATGC
	citZ_rev_T7	CTAATACGACTCACTATAGGGAGGGCTCAGCGTTTTCCACTTC
citB	citB_for_T7	GCGTTAGAAGATTCAGGTATAGG
	citB_rev_T7	CTAATACGACTCACTATAGGGAGTTGTGTTACTTTTAACGCCAAGTC
yclM	yclM_for_T7	AAAGCTGTAGTCGTTTCAGCT
	yclM_rev_T7	CTAATACGACTCACTATAGGGAGCGGATTGACAGAATACACAGC
hom	hom_for_T7	AGAGAAGTAGATTTGCCGAAGG
	hom_rev_T7	CTAATACGACTCACTATAGGGAGGCTGAAGCTGATGTCCTCG
yoaD	yoaD_for_T7	GGCTCAGTTGCTTATCAATCTTGG
	yoaD_rev_T7	CTAATACGACTCACTATAGGGAGGAGTGTTTCCTCAGTGCGAGG
gltA	gltA_for_T7	GGACTTAAGATGCTTTGCCAGC
	gltA_rev_T7	CTAATACGACTCACTATAGGGAGGCTTCGTCTTGCAGATCAGA
rpsP	rpsP_for_T7	GGCAGTAAAAATTCGTTTAAAACG
	rpsP_rev_T7	CTAATACGACTCACTATAGGGAGGCCTTGTTTAGCGTTGTGG
lpdV	lpdV_for_T7	CTGCATAAAGGCTGTATCCCG
	lpdV_rev_T7	CTAATACGACTCACTATAGGGAGCAGTCGGCAATATGCGATCC
yurO	yurO_for_T7	CGAGCCAAAGCAGTTCAGC
	yurO_for_T7	CTAATACGACTCACTATAGGGAGCGAGATTGTCCAAGTCGCC
ald	ald_for_T7	GAAGCGGATTTGAAAATGAAGC
	ald_rev_T7	CTAATACGACTCACTATAGGGAGGCCACAGCATCAGCAATATTG
dhbF	MMdhbF_for	TCGCTGCATGTCCGCTTCGG
	MMdhbF_rev_T7	CTAATACGACTCACTATAGGGAGCCGACCCATCATGGGCAGGC
feuA	MMfeuA_for	CGCGCTGACGGCGGCAGC
	MMfeu_rev_T7	CTAATACGACTCACTATAGGGAGACTTCGTTCGGCGCCTTAAGGCC